Plant Physiol. (1996) 111: 613–618

Specificity of the Organic Acid Activation of Alternative Oxidase in Plant Mitochondria[1]

A. Harvey Millar, Marcel H. N. Hoefnagel, David A. Day*, and Joseph T. Wiskich

Division of Biochemistry and Molecular Biology, and The Cooperative Research Centre for Plant Science, Australian National University, Canberra 0200, Australia (A.H.M., D.A.D.); and Botany Department, University of Adelaide, Adelaide 5005, South Australia, Australia (M.H.N.H., J.T.W.)

The claim that succinate and malate can directly stimulate the activity of the alternative oxidase in plant mitochondria (A.M. Wagner, C.W.M. van den Bergen, H. Wincencjusz [1995] Plant Physiol 108: 1035–1042) was reinvestigated using sweet potato (*Ipomoea batatas* L.) mitochondria. In whole mitochondria, succinate (in the presence of malonate) and both L- and D-malate stimulated respiration via alternative oxidase in a pH- (and NAD^+)-dependent manner. Solubilized malic enzyme catalyzed the oxidation of both L- and D-malate, although the latter at only a low rate and only at acid pH. In submitochondrial particle preparations with negligible malic enzyme activity, neither L- nor D-malate stimulated alternative oxidase activity. However, even in the presence of high malonate concentrations, some succinate oxidation was observed via the alternative oxidase, giving the impression of stimulation of the oxidase. Neither L-malate nor succinate (in the presence of malonate) changed the dependence of alternative oxidase activity on ubiquinone reduction state in submitochondrial particles. In contrast, a large change in this dependence was observed upon addition of pyruvate. Half-maximal stimulation of alternative oxidase by pyruvate occurred at less than 5 μM in submitochondrial particles, one-twentieth of that reported for whole mitochondria, suggesting that pyruvate acts on the inside of the mitochondrion. We suggest that malate and succinate do not directly stimulate alternative oxidase, and that reports to the contrary reflect intramitochondrial generation of pyruvate via malic enzyme.

In the inner membrane of plant mitochondria there are two pathways that oxidize reduced Q: the Cyt and alternative pathways. In the former, electron transport is coupled to ATP production, whereas the latter is nonphosphorylating and releases energy as heat. The physiological role of this alternative pathway may be to avoid overreduction of the electron transport chain and the subsequent production of reactive oxygen species in the cell (Minagawa et al., 1992; Purvis and Shewfelt, 1993). Stress conditions such as treatment with Cyt pathway inhibitors or cycloheximide, wounding, exposure to cold, and treatment with salicylic acid induce AOX synthesis (Minagawa and Yoshimoto, 1987; Hiser and McIntosh, 1990; Morohashi et al., 1991; Vanlerberghe and McIntosh, 1992a, 1992b; Rhoads and McIntosh, 1993). The activity of AOX is controlled by the oxidation or reduction of sulfhydryl groups, which form enzyme dimers (Umbach and Siedow, 1993), and this can be regulated in intact mitochondria by the redox poise of the NADP(H) pool in the mitochondrial matrix (Vanlerberghe et al., 1995).

AOX activity is also dependent on the presence of pyruvate and some other α-keto acids (Millar et al., 1993) that stimulate the oxidase and change its kinetics so that it can function at lower levels of reduction of the Q pool (Umbach et al., 1994; Hoefnagel et al., 1995). Since partially purified AOX from *Arum* and soybean (*Glycine max* [L.]) cotyledons is stimulated and stabilized by pyruvate (Zhang et al., 1996), it is obvious that pyruvate reacts directly with the AOX protein. In soybean, it has been shown that pyruvate generated intramitochondrially during oxidation of malate and succinate can also lead to activation of AOX (Day et al., 1994), explaining the frequent observation that these substrates are able to sustain higher rates of alternative pathway activity than the more rapidly oxidized substrate NADH (see Lance et al., 1985, for review).

However, it has also been postulated that succinate and malate in some way directly activate AOX in potato mitochondria. Succinate stimulated NADH oxidation even in the presence of the succinate dehydrogenase inhibitor malonate (Wagner et al., 1989; Lidén and Åkerlund, 1993). Wagner et al. (1989, 1995) showed that L-malate and its supposedly unmetabolized isomer, D-malate, stimulated AOX activity in potato callus mitochondria. On the basis of the work by Cheng et al. (1986) showing that malate and succinate could affect membrane fluidity, Wagner et al. (1989) postulated that these dicarboxylates acted by increasing lateral diffusion of substrates and protein complexes in the mitochondrial membrane, thereby increasing electron flux through AOX. Recently, stimulations by succinate and malate were shown to be temperature dependent in potato callus mitochondria, and a role for membrane fluidity in the action of these compounds was again postulated (Wagner et al., 1995). This is possible given that, in contrast to pyruvate, neither malate nor succinate stimulate AOX after isolation from the mitochondrial membrane (Zhang et al., 1996).

[1] This research was supported by grants from the Australian Research Council (D.A.D., J.T.W.) and an Australian Postgraduate Award and Student Assistantship from the Cooperative Research Centre for Plant Science (A.H.M.).

* Corresponding author; e-mail david.day@anu.edu.au; fax 61–6–249–0313.

Abbreviations: AOX, alternative oxidase; ME, malic enzyme; Q, ubiquinone; SMPs, inside-out submitochondrial particles.

Given the probable role of AOX in plant stress responses, it is important that all possible regulatory mechanisms be elucidated in detail. In this context, we have conducted a survey of the ability of a range of organic acids to stimulate AOX and have investigated the effects of malate and succinate in detail in sweet potato (*Ipomoea batatas* L.) mitochondria. Both whole mitochondria and SMPs were exploited to differentiate between direct activators and those that act via metabolism in the matrix. Simultaneous measurements of NADH oxidation and Q-pool reduction were used to monitor the kinetics of AOX upon the addition of putative effectors. We conclude that the observed effects of succinate and both isomers of malate (Wagner et al., 1995) can be explained by their metabolism to pyruvate in the mitochondrial matrix.

MATERIALS AND METHODS

Reagents

Percoll was purchased from Pharmacia. Q-1 was prepared by Dr. D. Ward (Organic Chemistry, Adelaide University, Australia) and reduced according to Rich (1981). All other reagents were purchased from Sigma.

Plant Materials

Soybeans (*Glycine max* [L.] Merr. cv Stevens) were grown under glasshouse conditions in trays of vermiculite, and after 14 d the cotyledons were harvested. Mitochondria were isolated as described by Day et al. (1985). Tubers of white sweet potato (*Ipomoea batatas* L. var Leucorrhiza) were purchased from local markets. Mitochondria were isolated according to Neuburger (1985) using self-generating Percoll gradients. Submitochondrial particles were prepared essentially by the method of Rasmusson and Møller (1991).

Assays

Oxygen uptake was measured using an oxygen electrode in 2 mL of standard reaction medium (containing 0.3 M Suc, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA, 10 mM Tes, pH varied from 6.5-7.5 as stated) at 25°C. The rate of NADH oxidation was determined spectrophotometrically at 340 nm. The level of Q reduction was monitored voltametrically, according to Moore et al. (1988), with titration of NADH oxidation as described previously (Hoefnagel and Wiskich, 1996). Malic enzyme activities were assayed as NADH production at 340 nm, according to Day et al. (1984), in a reaction medium of 2 mM NAD^+, 2 mM $MnCl_2$, 4 mM DTT, 0.02% (v/v) Triton TX-100, 1 μM antimycin A, 50 μM *n*-propylgallate, and 50 mM Mes/bis-tris-propane, pH 6.5 to 7.5. The protein content of samples was estimated by the method of Lowry et al. (1951). Unless otherwise stated, data presented in figures and tables represent typical results from one of three independent experiments.

RESULTS

Effect of Organic Acids on AOX

A variety of organic acids was tested for their effect on AOX activity (measured as myxothiazol-insensitive NADH oxidation) in sweet potato and/or soybean cotyledon mitochondria; only a few stimulated significantly and these were predominantly α-keto acids (Table I). Short-chained α-keto acids such as pyruvate were the most effective, acting at 0.1 to 0.5 mM, whereas higher concentrations of the longer-chained oxaloacetate and α-ketoglutarate were required (data not shown; see Millar et al., 1993; Vanlerberghe et al., 1995; Wagner et al., 1995). Since it appears that pyruvate acts from within the matrix (Day et al., 1994), these measurements in intact mitochondria are probably confounded by carrier kinetics on the mitochondrial inner membrane (Day and Hanson, 1977). In SMPs, the matrix face of the inner membrane is directly accessible to added organic acids and, in these particles, half-maximal stimulation by pyruvate occurred at concentrations less than 5 μM in both sweet potato and soybean (Fig. 1). This is additional evidence that the site of pyruvate action is on the inside of the inner mitochondrial membrane. Similar micromolar concentrations of glyoxylate and hydroxypyruvate were needed for activation in soybean SMPs, whereas several millimolar α-ketoglutarate were required (Fig. 1).

Some of the acids tested, however, did not contain the α-keto acid motif but nonetheless stimulated myxothiazol-insensitive NADH oxidation; these were the dicarboxylates L-malate and succinate (Table I). D-Malate and succinate + malonate (to inhibit succinate oxidation) did not stimulate

Table I. *The effects of various organic acids on NADH oxidation via the AOX in soybean cotyledon and/or sweet potato mitochondria*

AOX activity was measured as myxothiazol-insensitive (5 μM) and *n*-propylgallate-sensitive (50 μM) oxygen consumption in the presence of NADH (1 mM) in a standard reaction medium (pH 7.2). Organic acids were added at 10 mM after a steady rate in the presence of myxothiazol, except in the cases of pyruvate, hydroxypyruvate, and glyoxylate, which were added to a final concentration of 1 mM. –, No tricarboxylic acids tested stimulated AOX.

Acid	Stimulate AOX	No Effect on AOX
Monocarboxylic acids	Pyruvate	Lactate
	Hydroxypyruvate	PEP
	Glyoxylate	Propionate
		Acetate
		Gly
		Ala
		Crotonate
		Acrylate
		Acetoacetate
Dicarboxylic acids	α-Ketoglutarate	Malonate
	Oxaloacetate	Fumarate
	L-Malate	Oxalate
	Succinate	Tartrate
		D-Malate
Tricarboxylic acids	–	Citrate
		Isocitrate

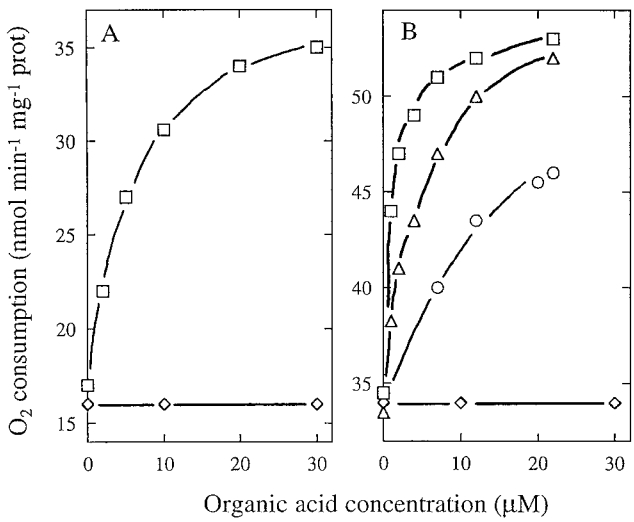

Figure 1. Concentration dependence of α-keto acid effects on NADH oxidation via the AOX in sweet potato and soybean cotyledon SMPs. The rate of NADH oxidation was measured as oxygen consumption in the presence of 5 μM myxothiazol in standard reaction medium. A, Sweet potato SMPs (0.6 mg of protein); B, soybean cotyledon SMPs (0.4 mg of protein). Pyruvate (□), glyoxylate (△), hydroxypyruvate (○), or α-ketoglutarate (◇) were added in increasing amounts to the final concentrations shown. prot, Protein.

AOX. Likewise, no stimulation of AOX activity was observed by 10 mM concentrations of the remaining mono-, di-, and tricarboxylic acids tested (Table I). Since these results contradicted those of Wagner et al. (1995), we investigated the effects of malate and succinate further.

Malate and Succinate Stimulation of AOX Is pH Dependent

A difference in protocol between our experiments and those of Wagner et al. (1995) is that we used a more alkaline pH; the effect of pH was consequently investigated in more detail. As shown in Figure 2, the rate of NADH oxidation in the presence of myxothiazol increased upon the addition of pyruvate at both pH 6.5 and 7.5. At pH 6.5, both D- and L-malate and succinate (plus malonate) also stimulated both oxygen uptake and NADH oxidation 2.5- to 5-fold in the presence of myxothiazol (Fig. 2A). In contrast, at pH 7.5 only L-malate and pyruvate caused a significant increase in NADH-dependent oxygen uptake (Fig. 2B). These results were also observed with ubiquinol-1 as substrate, but with this substrate D- and L-malate stimulated only after addition of 0.1 mM NAD^+ (not shown).

ME Oxidizes Both L- and D-Malate

The activity of NAD-ME is strongly inhibited under alkaline conditions in plant mitochondria (Macrae, 1971; Tobin et al., 1980). We investigated ME activity in sweet potato mitochondria at different pH values with both D- and L-malate as substrates (Table II). At pH 6.5, both D- and L-malate were effective as substrates, although the latter was oxidized 10-fold more rapidly. At pH 7.5, little measurable activity was observed with D-malate, whereas L-malate oxidation was reduced by 70 to 90%, depending on the presence of the activator CoA (Table II). The ME activity of sweet potato at pH 6.5 was slightly lower than that reported in potato (300-400 nmol min^{-1} mg^{-1} protein; Day et al., 1984), but substantially higher than that reported for soybean root and cotyledon mitochondria (40-150 nmol min^{-1} mg^{-1} protein; Day and Mannix, 1988; Day et al., 1994).

These results suggest that D-malate and succinate in the presence of malonate can be metabolized by sweet potato mitochondria. This is supported by the effects of these compounds on Q redox state (Fig. 3). In the presence of myxothiazol, 10 mM succinate increased the level of Q reduction (Qr/Qt) from 0.12 to 0.35, despite the presence of 25 mM malonate, and D-malate also increased Qr/Qt. In both cases the rate of oxygen consumption was still negligible. In the control (Fig. 3A), Qr/Qt reached 0.78 in the

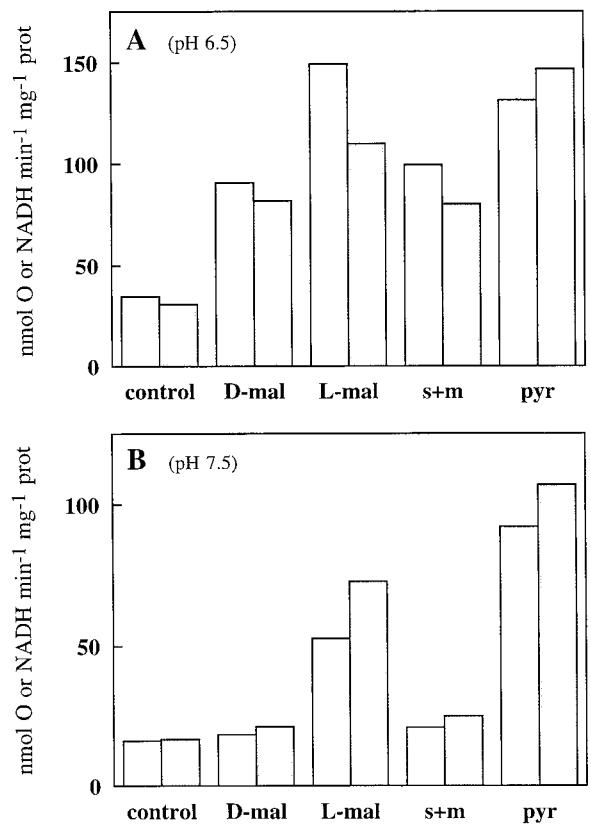

Figure 2. Effects of organic acids on the NADH oxidation via the AOX in sweet potato mitochondria. The rate of NADH oxidation at pH 6.5 (A) or 7.5 (B) was measured as oxygen uptake using an oxygen electrode (open bar) and as NADH consumption (shaded bar). Sweet potato mitochondria (0.6 mg of protein) were incubated in standard reaction medium in the presence of 5 μM myxothiazol. To the control the following acids were added in separate assays: 10 mM D- and L-malate (D- and L-mal), 10 mM succinate (in the presence of 25 mM malonate) (s+m), and 1 mM pyruvate (pyr). The average control rate (in the presence of myxothiazol) is shown, and the other rates were normalized to allow for representation in a graph. The uninhibited rate of NADH oxidation was 131 and 85 nmol O_2 min^{-1} mg^{-1} protein at pH 6.5 and 7.5 respectively. prot, Protein.

Table II. *Dependence of ME activity on pH in sweet potato mitochondria with L-malate or D-malate as substrate*

Rates of ME activity of sweet potato mitochondria (0.2 mg protein) utilizing 10 mM L-malate or 10 mM D-malate measured as NADH production at 340 nm on a spectrophotometer in a reaction medium containing: 2 mM NAD^+, 2 mM $MnCl_2$, 4 mM DTT, 0.02% (v/v) Triton TX-100, 1 μM antimycin A, 50 μM *n*-propylgallate, and 50 mM Mes/bis-tris-propane, pH 6.5 to 7.5. Rates in the presence and absence of 25 μM CoA are presented, $n = 3$.

Substrate	pH 6.5	pH 7.5
	nmol NADH min^{-1} mg^{-1} protein	
L-Malate		
−CoA	255 ± 40	10 ± 9
+CoA	298 ± 38	109 ± 10
D-Malate		
−CoA	16 ± 1	2 ± 0.2
+CoA	37 ± 4	2 ± 0.2

presence of succinate and myxothiazol with substantial oxygen consumption. This is consistent with the steep kinetics of AOX activity with respect to Q. An increase in Q reduction was also observed in submitochondrial particles, with succinate + malonate but not with D-malate (data not shown). That is, some oxidation of succinate by succinate dehydrogenase occurred even when malonate was present at high concentrations.

Stimulation of AOX in SMPs

To eliminate the possible conversion of D-malate and succinate to pyruvate via ME, SMPs, which are inside-out inner-membrane vesicles and largely free of soluble matrix enzymes, were employed. ME activity in sweet potato SMPs was negligible (not shown). In these SMPs, D- and L-malate failed to stimulate NADH oxidation through AOX at either pH 6.5 or 7.5 (Fig. 4), whereas pyruvate stimulated the rates of both oxygen uptake and NADH oxidation. Succinate + 25 mM malonate, on the other hand, caused a slight increase in the rate of oxygen uptake at both pH values (Fig. 4). However, the rate of NADH oxidation increased only slightly at pH 6.5 and remained unchanged at pH 7.5, indicating that the effect of succinate was mostly due to it acting as a substrate, despite the presence of the (competitive) inhibitor malonate. This idea is consistent with the partial reduction of the Q pool observed on addition of succinate in the presence of 25 mM malonate and myxothiazol (Fig. 3).

An alternative explanation for the results with SMPs is that, unlike pyruvate (see above), malate and succinate act on the outside of the inner membrane and cannot gain access to this site in SMPs. This is unlikely, however, because the dicarboxylate carrier on the inner membrane catalyzes dicarboxylate exchange in both directions (DeSantis et al., 1976).

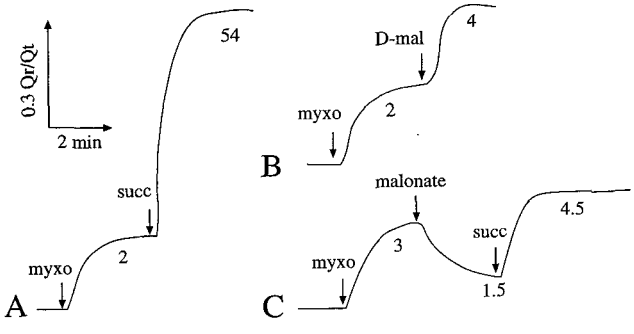

Figure 3. Effect of organic acids on Q redox state. Oxygen consumption and Q reduction were measured simultaneously on addition of succinate (A), D-malate (B), and succinate after malonate (C) in sweet potato mitochondria in the presence of myxothiazol at pH 6.5. Where indicated, 5 μM myxothiazol (myxo), 10 mM succinate (succ), 10 mM D-malate (D-mal), and 25 mM malonate were added. Numbers on traces are nmol O_2 min^{-1} mg^{-1} protein. Upward deflection of trace indicates Q reduction.

The Effects of Organic Acids on Qr/Qt versus AOX Activity in SMPs

The effect of pyruvate and other α-keto acids has been most clearly demonstrated by their effect on the relation-

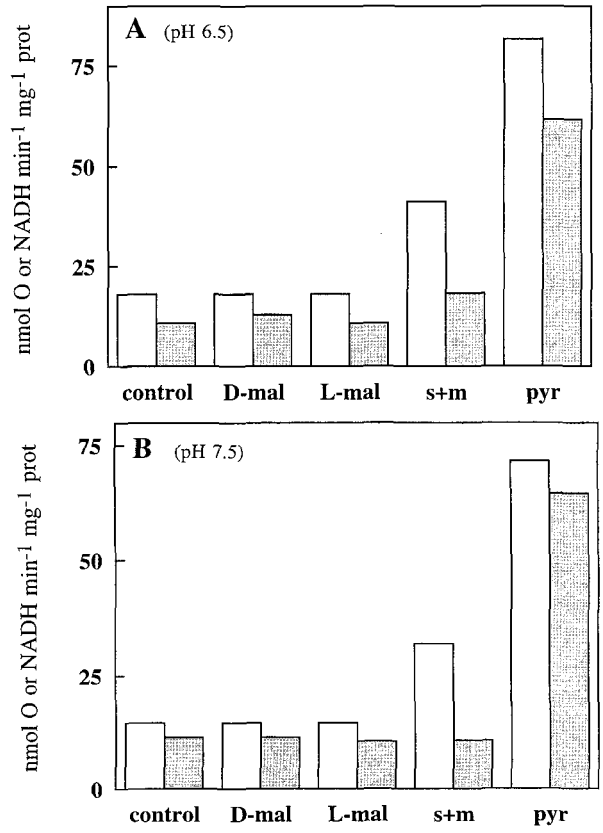

Figure 4. Effects of organic acids on the NADH oxidation via the AOX in sweet potato SMPs. The rate of NADH oxidation at pH 6.5 (A) or pH 7.5 (B) was measured as oxygen uptake using an oxygen electrode (open bar) and as NADH consumption (shaded bar). Sweet potato SMPs (0.21 mg of protein) were incubated in standard reaction medium in the presence of 5 μM myxothiazol. To the control the following acids were added in separate assays: 10 mM D- and L-malate (D- and L-mal), 10 mM succinate (in the presence of 25 mM malonate) (s+m), and 1 mM pyruvate (pyr). The average control rate (in the presence of myxothiazol) is shown, and the other rates were normalized to allow for representation in a graph. prot, Protein.

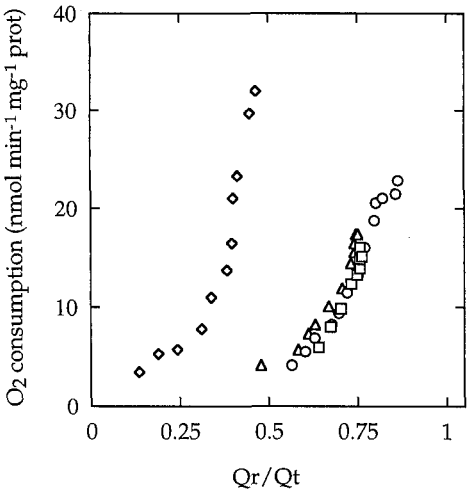

Figure 5. Dependence of AOX activity on Q redox state in sweet potato SMPs oxidizing NADH. The rate of NADH oxidation was measured as oxygen consumption in the presence of 5 μM myxothiazol in standard reaction medium (pH 7.2). Titrations on SMPs (1.12 mg of protein) with increasing NADH: control (□) in the presence of 1 mM pyruvate (◇), 10 mM succinate and 25 mM malonate (○), or 10 mM L-malate (△). prot, Protein.

ship between AOX activity and reduction of the Q pool (Umbach et al., 1994). Therefore, we tested the effect of succinate and L-malate on this relationship in SMPs, where organic acid metabolism is effectively eliminated by the removal of matrix enzymes. Using this approach, the dual roles of succinate as both substrate and putative activator can be distinguished. Neither L-malate nor succinate + malonate had a significant effect on the dependence of AOX activity on the Q reduction level in sweet potato SMPs (Fig 5; similar results were obtained with soybean). Pyruvate, on the other hand, activated AOX and shifted the Qr/Qt curve to the left, just as it does in soybean mitochondria (Umbach et al., 1994; Day et al., 1995). Neither malonate, succinate, nor malate affected the pyruvate stimulation (determined by the stimulation of oxygen uptake by pyruvate when added at the end of the NADH titrations shown in Fig. 5). In the presence of succinate + malonate, higher Q reduction levels were attained than in the control (Fig. 5), again suggesting that succinate oxidation is not fully inhibited by the competitive inhibitor malonate at this concentration. In a separate experiment it was found that addition of succinate to sweet potato SMPs oxidizing NADH in the presence of 25 mM malonate increased Q reduction from 93 to 95%, and oxygen uptake increased by 20%.

DISCUSSION

The results presented here confirm that organic acid stimulation of AOX activity in plant mitochondria is confined to a narrow range of α-keto acids. Our results also show: (a) that pyruvate is effective at much lower concentrations when the inner face of the inner membrane is exposed than in intact mitochondria, suggesting that it acts within the matrix; (b) that plant mitochondria can slowly metabolize succinate via succinate dehydrogenase, even when malonate is present, and can metabolize D-malate via ME; and (c) that succinate and malate do not stimulate AOX activity when care is taken to eliminate ME activity.

Clearly, malate and succinate are not direct activators of AOX, and previously reported effects of these compounds on AOX activity in whole mitochondria were, quite likely, the result of their conversion to pyruvate (Fig. 6). The very small concentrations of pyruvate needed to stimulate AOX (Fig. 1) and the high levels of ME in mitochondria from sweet potato (Table II) and potato (Day et al., 1984) exacerbate this effect, especially when respiration is measured at acid pH, as was the case in the studies by Wagner et al. (1989, 1995) and Lidén and Åkerlund (1993). The latter authors recognized the need for succinate and malate to be metabolized to achieve their stimulating effect on AOX.

In SMPs there was a small stimulation of oxygen uptake by succinate + malonate (Fig. 4), which was probably the result of an increase in the level of reduction of the Q pool (Fig. 3). The titrations of the redox level of Q with NADH as a substrate (Fig. 5) showed that the dependence of AOX activity on the level of Q reduction did not really change in the presence of succinate, demonstrating that the effect of succinate was to act as a substrate rather than as an activator. Similar results have been obtained with *Arum* mitochondria (Hoefnagel and Wiskich, 1996).

Membrane Fluidity and AOX Activation

Malate, succinate, and pyruvate stimulation of AOX have been shown to be temperature dependent in potato callus mitochondria, with little stimulation seen below 17 to 20°C (Wagner et al., 1995). It was postulated that these organic acids might activate AOX through membrane fluidity changes (Wagner et al., 1989), although no such effects on membrane fluidity could be detected (Wagner et al., 1995). In soybean cotyledon mitochondria, no temperature dependence of pyruvate stimulation was observed between 4 and 28°C (A.H. Millar, unpublished data). Likewise, no temperature dependence was observed in sweet

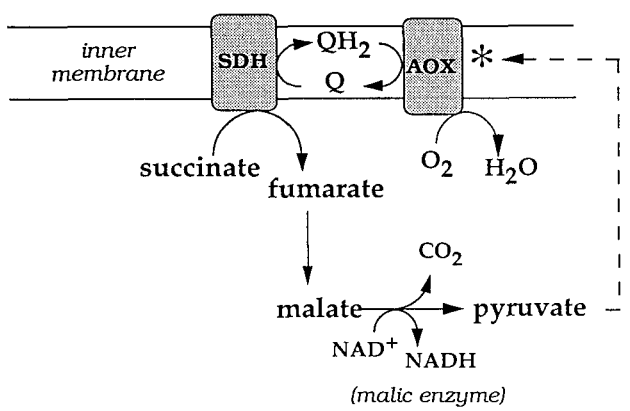

Figure 6. Stimulation of AOX in plant mitochondria by the dicarboxylates malate and succinate through electron donation to the Q pool and metabolism to pyruvate in the matrix. SDH, Succinate dehydrogenase. Asterisk and dashed arrow, Activation by pyruvate; solid arrow, reaction pathways.

potato mitochondria (Wagner et al., 1995). Because the solubilized, partially purified enzyme from *Arum* is also activated by pyruvate (Zhang et al., 1996), it is most unlikely that membrane fluidity changes can account for the activation of AOX by α-keto acids.

CONCLUSION

From the present study with sweet potato mitochondria and earlier studies with other species we conclude that activators of AOX appear to be restricted to α-keto acids. Small α-keto acids (C2-3) are most effective, and of these, the physiologically most important is probably pyruvate, although glyoxylate and hydroxypyruvate are of interest in the context of photorespiration. Stimulation by longer-chain α-keto acids, such as α-ketoglutarate (C5), requires much higher concentrations. Since pyruvate acts at very low concentrations, the use of SMPs or purified enzyme preparations to avoid metabolism is highly recommended when assessing the ability of compounds to activate AOX.

Received January 16, 1996; accepted March 20, 1996.

LITERATURE CITED

Cheng B, Feng Y, Lin K (1986) Effects of substrates and inhibitors for respiratory chain on membrane fluidity of mitochondrial inner membrane. Shengwu Hauaxau Zazhi **2:** 37–43

Day DA, Hanson JB (1977) Pyruvate and malate transport and oxidation in corn mitochondria. Plant Physiol **59:** 630–635

Day DA, Mannix M (1988) Malate oxidation by soybean nodule mitochondria and the possible consequences for nitrogen fixation. Plant Physiol Biochem **26:** 567–573

Day DA, Millar AH, Wiskich JT, Whelan J (1994) Alternative oxidase regulation by pyruvate in soybean. Plant Physiol **106:** 1421–1427

Day DA, Neuburger M, Douce R (1984) Activation of NAD-linked malic enzyme in intact plant mitochondria by exogenous coenzyme A. Arch Biochem Biophys **231:** 233–242

Day DA, Neuburger M, Douce R (1985) Biochemical characterisation of chlorophyll-free mitochondria from pea leaves. Aust J Plant Physiol **12:** 219–228

Day DA, Whelan J, Millar AH, Siedow JN, Wiskich JT (1995) Regulation of the alternative oxidase in plants and fungi. Aust J Plant Physiol **22:** 497–509

DeSantis A, Arrigoni O, Palmieri F (1976) Carrier-mediated transport of metabolites in purified bean mitochondria. Plant Cell Physiol **17:** 1221–1233

Hiser C, McIntosh L (1990) Alternative oxidase of potato is an integral membrane protein synthesized de novo during aging of tuber slices. Plant Physiol **93:** 312–318

Hoefnagel MHN, Millar AH, Wiskich JT, Day DA (1995) Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. Arch Biochem Biophys **318:** 394–400

Hoefnagel MHN, Wiskich JT (1996) Alternative oxidase activity and the ubiquinone redox level in soybean cotyledon and *Arum* spadix mitochondria during NADH and succinate oxidation. Plant Physiol **110:** 1329–1335

Lance C, Chaveau M, Dizengremel P (1985) The cyanide-resistant pathway of plant mitochondria. *In* R Douce, DA Day, eds, Higher Plant Cell Respiration: Encyclopedia of Plant Physiology (New Series), Vol 18. Springer-Verlag, Berlin, pp 202–247

Lidén AC, Åkerlund H-E (1993) Induction and activation of the alternative oxidase of potato tuber mitochondria. Physiol Plant **87:** 134–141

Lowry O, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem **193:** 265–275

Macrae AR (1971) Isolation and properties of malic enzyme from cauliflower bud mitochondria. Biochem J **122:** 495–501

Millar AH, Wiskich JT, Whelan J, Day DA (1993) Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett **329:** 259–262

Minagawa N, Koga S, Nakano M, Sakajo S, Yoshimoto A (1992) Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala.* FEBS Lett **302:** 217–219

Minagawa N, Yoshimoto A (1987) The induction of cyanide-resistant respiration in *Hansenula anomala.* J Biochem **101:** 1141–1146

Moore AL, Dry IB, Wiskich JT (1988) Measurement of the redox state of the ubiquinone pool in plant mitochondria. FEBS Lett **235:** 76–80

Morohashi Y, Seto T, Matsushima H (1991) Appearance of alternative respiration in cucumber cotyledon mitochondria after treatment with cycloheximide. Physiol Plant **83:** 640–646

Neuburger M (1985) Preparation of plant mitochondria, criteria for assessment of mitochondrial integrity and purity, survival in vitro. *In* R Douce, DA Day, eds, Higher Plant Cell Respiration: Encyclopedia of Plant Physiology (New Series), Vol 18. Springer-Verlag, Berlin, pp 7–24

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol Plant **88:** 712–718

Rasmusson AG, Møller IM (1991) NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane studied using inside-out sub-mitochondrial particles. Physiol Plant **83:** 357–365

Rhoads DM, McIntosh L (1993) Salicylic acid-inducible alternative oxidase gene *aox1* and genes encoding pathogenesis-related proteins share regions of sequence similarity in their promoters. Plant Mol Biol **21:** 615–624

Rich PR (1981) Quinol oxidation in *Arum maculatum* mitochondria and its application to the assay, solubilisation and partial purification of the alternative oxidase. FEBS Lett **96:** 252–256

Tobin A, Djerdjour B, Journet E-P, Neuburger M, Douce R (1980) Effect of NAD on malate oxidation in intact plant mitochondria. Plant Physiol **66:** 225–229

Umbach AL, Siedow JN (1993) Covalent and non-covalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol **103:** 845–854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox state in soybean seedling mitochondria. FEBS Lett **348:** 181–184

Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE, McIntosh L (1995) Alternative oxidase activity in tobacco leaf mitochondria. Dependence on tricarboxylic acid cycle-mediated redox regulation and pyruvate activation. Plant Physiol **109:** 353–361

Vanlerberghe GC, McIntosh L (1992a) Coordinate regulation of cytochrome and alternative pathway respiration in tobacco. Plant Physiol **100:** 1846–1851

Vanlerberghe GC, McIntosh L (1992b) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco callus. Plant Physiol **100:** 115–119

Wagner AM, Kraak MS, van Emmerik WAM, van der Plas LHW (1989) Respiration of plant mitochondria with various substrates: alternative pathway with NADH and TCA cycle derived substrates. Plant Physiol Biochem **27:** 837–845

Wagner AM, van den Bergen CWM, Wincencjusz H (1995) Stimulation of the alternative pathway by succinate and malate. Plant Physiol **108:** 1035–1042

Zhang Q, Hoefnagel MHN, Wiskich JT (1996) Alternative oxidase in *Arum* and soybean: its stabilization during purification. Physiol Plant **95:** (in press)